Development 133, 4173-4181 (2006) doi:10.1242/dev.02577

Repression of mesodermal fate by *foxa*, a key endoderm regulator of the sea urchin embryo

Paola Oliveri[1,*], Katherine D. Walton[2], Eric H. Davidson[1] and David R. McClay[2,*]

The *foxa* gene is an integral component of the endoderm specification subcircuit of the endomesoderm gene regulatory network in the *Strongylocentrotus purpuratus* embryo. Its transcripts become confined to veg2, then veg1 endodermal territories, and, following gastrulation, throughout the gut. It is also expressed in the stomodeal ectoderm. *gatae* and *otx* genes provide input into the pregastrular regulatory system of *foxa*, and Foxa represses its own transcription, resulting in an oscillatory temporal expression profile. Here, we report three separate essential functions of the *foxa* gene: it represses mesodermal fate in the veg2 endomesoderm; it is required in postgastrular development for the expression of gut-specific genes; and it is necessary for stomodaeum formation. If its expression is reduced by a morpholino, more endomesoderm cells become pigment and other mesenchymal cell types, less gut is specified, and the larva has no mouth. Experiments in which blastomere transplantation is combined with *foxa* MASO treatment demonstrate that, in the normal endoderm, a crucial role of Foxa is to repress *gcm* expression in response to a Notch signal, and hence to repress mesodermal fate. Chimeric recombination experiments in which veg2, veg1 or ectoderm cells contained *foxa* MASO show which region of *foxa* expression controls each of the three functions. These experiments show that the *foxa* gene is a component of three distinct embryonic gene regulatory networks.

KEY WORDS: Sea urchin, *foxa*, Forkhead, Regulatory network, Endomesoderm, Specification

INTRODUCTION

The endomesoderm gene regulatory network (GRN) models the transcriptional control system defining vegetal specification of the sea urchin (*Strongylocentrotus purpuratus*) embryo during the first 30 hours of development. The GRN is a nuclear view of transcriptional regulation and incorporates data from many sources. In addition, it includes known signal transduction events that subdivide the endomesoderm into subnetwork modules during this initial 30-hour period. By gastrulation, mesoderm and endoderm have been defined, and have acquired competence to undergo the morphogenetic movements of gastrulation. The network model further provides the genomic regulatory code for subsequent steps of differentiation and morphogenesis.

The GRN is activated by maternal inputs at the vegetal pole, first seen as nuclearization of β-catenin in the micromeres and in macromeres (Logan et al., 1999). Between the fourth and sixth cleavage, the early signal, the molecular nature of which is not yet known, from the micromeres provides added input to the macromeres to accelerate endomesoderm GRN activation (Ransick and Davidson, 1995; Oliveri et al., 2003). At sixth cleavage, the veg2 tier of endomesoderm cells separates from their sister veg1 cells, the veg2 tier occupying a more vegetal position. Between the seventh and ninth cleavages, the innermost veg2 cells receive a Delta signal from the adjacent micromeres, and the Notch-activated cells become secondary mesenchyme (SMC) precursors. This Notch (N) signal functionally distinguishes SMC from endoderm specification (Sherwood and McClay, 1999). A second Delta signaling event later originates in the SMC precursors, contributes to SMC specification, and is required to define the adjacent veg2 endoderm subcompartment (Sherwood and McClay, 1999; Sweet et al., 2002; Peterson and McClay, 2005). In the sea urchin embryo, at least 16 transcription factors are activated specifically in cells that become endoderm, and among these is the *foxa* gene.

Foxa belongs to the forkhead family of transcription factors. Orthologous genes have been isolated in many different species, including *Drosophila* (Weigel et al., 1989), *C. elegans* (Mango et al., 1994), mouse (Ang et al., 1993), ascidians (Corbo et al., 1997; Olsen and Jeffery, 1997), hemichordates (Taguchi et al., 2000) and cnidarians (Fritzenwanker et al., 2004; Martindale et al., 2004). A common feature is that Foxa factors are restricted to endodermal cells just prior to, or during, gastrulation, and are necessary for the specification and differentiation of endodermal structures. Later, these factors are required in other domains and other developing structures. In *Xenopus*, *foxa2/hnf3β* is initially expressed at the vegetal pole of the embryo, but is excluded from the mesoderm during gastrulation (Suri et al., 2004). In this embryo during gastrulation, *foxa2/hnf3β* has the major role of determining the mesodermal boundary by repressing mesoderm fate in the endoderm.

Sea urchin *foxa* was originally identified in *Hemicentrotus pulcherrimus* and named *hnf3* (Harada et al., 1996). The nomenclature used here is current for Forkhead transcription factors of this class (Kaestner et al., 2000). In situ hybridization showed *Hpfoxa* to be expressed initially in the vegetal plate, then surrounding the blastopore and finally in the gut of the embryo. Here, we characterize the functional role of *foxa* in the endomesoderm regulatory network by adding a high-resolution analysis of the *foxa* expression pattern in both *S. purpuratus* and *Lytechinus variegatus*, by performing experimental perturbations and embryological manipulations, and by using biochemical approaches to show that *foxa* has at least three major roles in the embryo: it assures that veg2 endoderm cells do not express mesoderm genes; it is required in the stomodeal region of the oral ectoderm for the production of the mouth; and it provides a controlling function for postgastrular development of the gut.

[1]Division of Biology, 156-29, California Institute of Technology, Pasadena, CA 91125, USA. [2]Department of Biology, Duke University, Durham, NC 27707, USA.

*Authors for correspondence (e-mail: poliveri@caltech.edu; dmcclay@duke.edu)

Accepted 9 August 2006

MATERIALS AND METHODS

Isolation and sequence analysis of *foxa* clones

A 224 bp fragment of *Spfoxa* cDNA (from position 644 to 867 of the GenBank sequence, Accession number DQ459376), corresponding to the most conserved region of the forkhead domain (a kind gift of Cathy Yuh, Division of Biology, Caltech) was used as probe for screening *Strongylocentrotus purpuratus* and *Lytechinus variegatus* bacterial artificial chromosome (BAC) genomic libraries. The 150 kb 41I19 *S. purpuratus* clone (GenBank Accession number AC131450) and the 55 kb *L. variegatus* clone 4G18 (GenBank Accession number AC131500) were sequenced by the Joint Genome Institute and the Institute for Systems Biology, respectively. Sequences are publicly available at http://sugp.caltech.edu/resources/annotation.psp.

Whole-mount in situ hybridization (WMISH)

Whole-mount in situ hybridization was performed as previously described (Minokawa et al., 2004). An RNA probe for *foxa* was synthesized in vitro from 200 ng of PCR fragment amplified from the 224 bp cDNA clone using the primers pSport F (5′-GTG CTG CAA GGC GAT TAA GT-3′) and pSport R (5′-TGT GGA ATT GTG AGC GGA TA-3′). For *gcm* probe description, see Ransick et al. (Ransick et al., 2002), and for *gatae* probe, see Lee and Davidson (Lee and Davidson, 2004).

Morpholino oligo antisense and mRNA injection constructs

A morpholino antisense substituted oligonucleotide (MASO) was synthesized (Gene Tools) complementary to the sequence just upstream of the first possible methionine, namely 5′-TGGGTTCCTCTTTGAAA-TCCACGAT-3′. A morpholino standard control 5′-CCTCTTACCTCA-gTTACAATTTATA-3′ was provided by Gene Tools. MASOs were injected in a 120 mM KCl solution, at final concentrations of 50 mM to 300 mM. The *foxa* coding construct was made using a vector derived from BlueScript (Statagene, La Jolla, CA), which contained the 3′UTR and 5′UTR of the globin gene (Lemaire et al., 1995). A fragment containing the entire coding sequence of the *foxa* gene was obtained by PCR, using the primers Foxa Cod F (5′-CATACACATCAGTGGAGGCT-3′) and Foxa Cod R (5′-TCC-ATCTATAACTGGTCGTG-3′). The 1659 bp PCR fragment was initially subcloned in pGEM-T easy vector (Promega). A *foxa* pGEM-T positive clone was digested with *Eco*RI to release the 1659 bp fragment with ends compatible with the *Eco*RI cloning site of the pBlueScript derived vector. The orientation of the cloned fragments was tested by PCR. To build the 5′*foxa*-GFP construct, a 540 bp fragment containing 150 bp of 5′UTR and 390 bp coding sequence was amplified by PCR, using the primers Foxa *Hin*dIII R (5′-CCCCAAGCTTGATACGATCAATAGA-3′) and Foxa *Kpn*I F (5′CCCCGGTACCAGGCTGACACTATATACT-3′). The GFP coding sequence was subcloned in frame as described by Oliveri et al. (Oliveri et al., 2002). The template used for these PCR reactions was the BAC clone 41I19. Each construct was checked by sequencing. RNA used for injection was synthesized as described by Oliveri et al. (Oliveri et al., 2002). The injection solutions were concentrated to 20 ng/μl, 50 ng/μl, or as indicated, and the RNA was injected together with fluorescein dextran or rhodamine dextran (10 pg/pl; Sigma).

Embryo manipulation and imaging

Embryo cultures of *L. variegatus* or *S. purpuratus*, and microsurgical procedures, were carried out as described earlier (McClay, 2000; Oliveri et al., 2003; Sweet et al., 2004). *L. variegatus* embryos were placed into Kiehart chambers in calcium-free sea water, whereas *S. purpuratus* embryos were placed in calcium-free sea water after two washes in Hyaline Extraction Medium. After surgery the embryos were returned to sea water.

Quantitative PCR (QPCR)

Total RNA was isolated from batches (100-200) of embryos injected with different MASOs and/or mRNA. The RNA was extracted using the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. First-strand cDNA was synthesized using random hexamers and the Taq Man Kit (PE Biosystems), as described by the manufacturer. The cDNA was used directly for quantitative PCR (QPCR) analysis. QPCR was conducted as previously described (Rast et al., 2000). For all QPCR experiments, the data from each cDNA sample were normalized against ubiquitin mRNA levels, which are known to remain relatively constant during development (Nemer et al., 1991; Oliveri and Davidson, 2004a; Ransick et al., 2002). The primers used can be found on the website http://sugp.caltech.edu/resources/methods/q-pcr.psp and have been previously published by Davidson et al. (Davidson et al., 2002).

RESULTS

Isolation and sequence analysis of the *foxa* gene

The *S. purpuratus* and *L. variegatus foxa* genes were identified and sequenced as described in the Materials and methods. In both species, the *foxa* gene consists of a single exon. A search for the *foxa* gene in the recently available *S. purpuratus* genome sequence showed that *foxa* is a single copy gene, as was previously determined for *Hemicentrotus pulcherrimus foxa* (Harada et al., 1996).

Sea urchin Foxa is a class A forkhead transcription factor. Predicted amino acid sequences for the sea urchin Foxa proteins are 98.1% identical between *S. purpuratus* and *H. pulcherrimus*, 94.5% identical between *S. purpuratus* and *L. variegatus*, and 95% identical between *L. variegatus* and *H. pulcherrimus*, over the whole length of the protein. There is thus no doubt that these genes are true orthologs. The phylogenetic relationship of *Spfoxa*, and thus *Lvfoxa*, to other forkhead class transcription factors has been resolved by Tu et al. (Tu et al., 2006).

Dynamic spatial expression of the sea urchin *foxa* gene

The analysis previously performed on *H. pulcherrimus* (Harada et al., 1996) was repeated to provide a higher resolution whole-mount in situ hybridization (WMISH) expression of *foxa* (Fig. 1). At 18 hours of development, *foxa* is expressed at a low level in the veg2 endomesoderm; the micromeres are devoid of expression (Fig. 1A,B). By 21 hours, expression of *foxa* becomes restricted

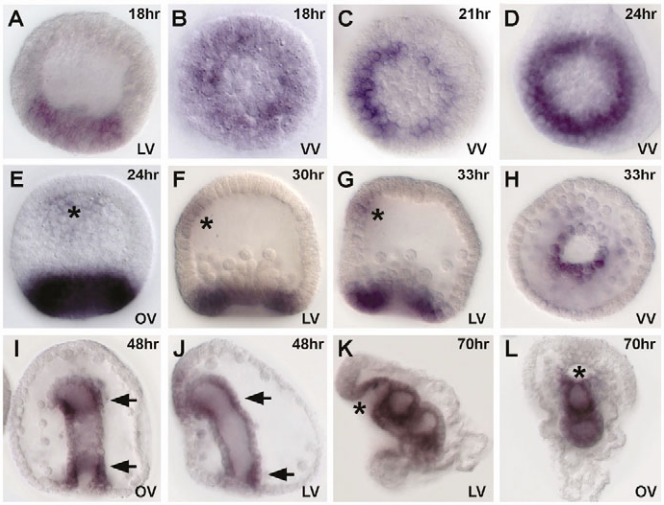

Fig. 1. Spatial expression pattern of *Spfoxa*. (**A-L**) WMISH was performed at seven different developmental stages, as indicated in the upper right corner of each panel. Note that starting at 21 hours postfertilization, asymmetric expression of *foxa* is observed across the endoderm (C,H,L). This is not as apparent in D, which is purposely overdeveloped to reveal that only endoderm expresses *foxa*. The asterisks in E-G,K,L indicate the stomodeal region of the oral ectoderm. Arrows (I,J) indicate higher expression levels in foregut and hindgut relative to the midgut. LV, lateral view; VV, view from the vegetal plate; OV, view from the oral ectoderm.

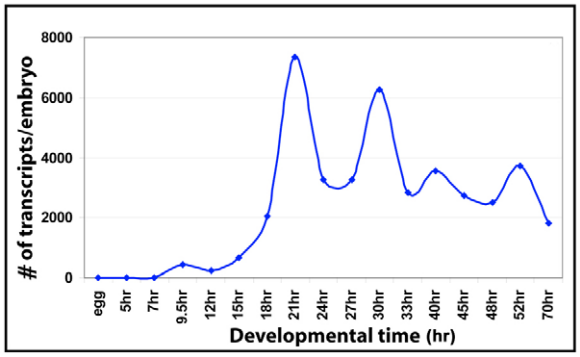

Fig. 2. Temporal expression of the *Spfoxa* gene in embryonic development. QPCR data obtained from different time points were converted to number of transcripts per embryo, by reference to a known standard (see Materials and methods). The transcript prevalence describes an oscillating pattern of expression with a period of 10±1 hours.

to the endodermal ring (Ruffins and Ettensohn, 1996). The gene is transcribed unequally in the two sides of the endodermal ring (Fig. 1C). At 21 hours and beyond (asterisk in Fig. 1E-G), the oral side of the endoderm, marked by the stomodeal expression, expresses a higher level of *foxa* than does the aboral side. The stomodeal expression domain was not identified in the previous analysis (Harada et al., 1996). Following primary mesenchyme cell (PMC) ingression (Fig. 1D), the endoderm expresses the gene at a high level. Thus, initially there is a transient expression in the endomesoderm, some of which will be specified as muscle and coelomic pouch SMCs, then, later, *foxa* is exclusively an endodermal and stomodeal gene.

A temporal expression profile at a 3-hour resolution is shown in Fig. 2. The *foxa* gene is not expressed maternally. Its zygotic expression begins at about 15 hours postfertilization, and continues throughout embryogenesis. The temporal expression profile displays a striking oscillatory periodicity, as has also been described for the starfish *foxa* gene (Hinman et al., 2003).

Conversion of endoderm to mesoderm on interference with *foxa* expression

A morpholino antisense substituted oligonucleotide (MASO) complementary to the translational start site of both the *S. purpuratus* and *L. variegatus foxa* mRNA was tested for efficacy in arresting translation using a GFP construct (5′*foxa*-GFP), which contained in-frame the target site sequence of the gene. As illustrated in Fig. 3J,K, the *foxa* MASO very effectively blocks the translation of RNA containing the initial ATG of the *foxa* message, but fails to block the translation of an altered form (5 base changes) that no longer recognizes the MASO (data not shown). Two different concentrations of *foxa* MASO were then injected into fertilized eggs. The embryonic phenotypes observed were of increasing severity at higher concentrations, and were equivalent in both species. Up to PMC ingression, the *foxA* MASO-injected embryos appeared normal, but at gastrulation MASO treatment caused a delay of gut invagination, and, at increased concentrations, a complete failure of invagination (Fig. 3A,B). If invagination occurred in MASO-treated embryos, the

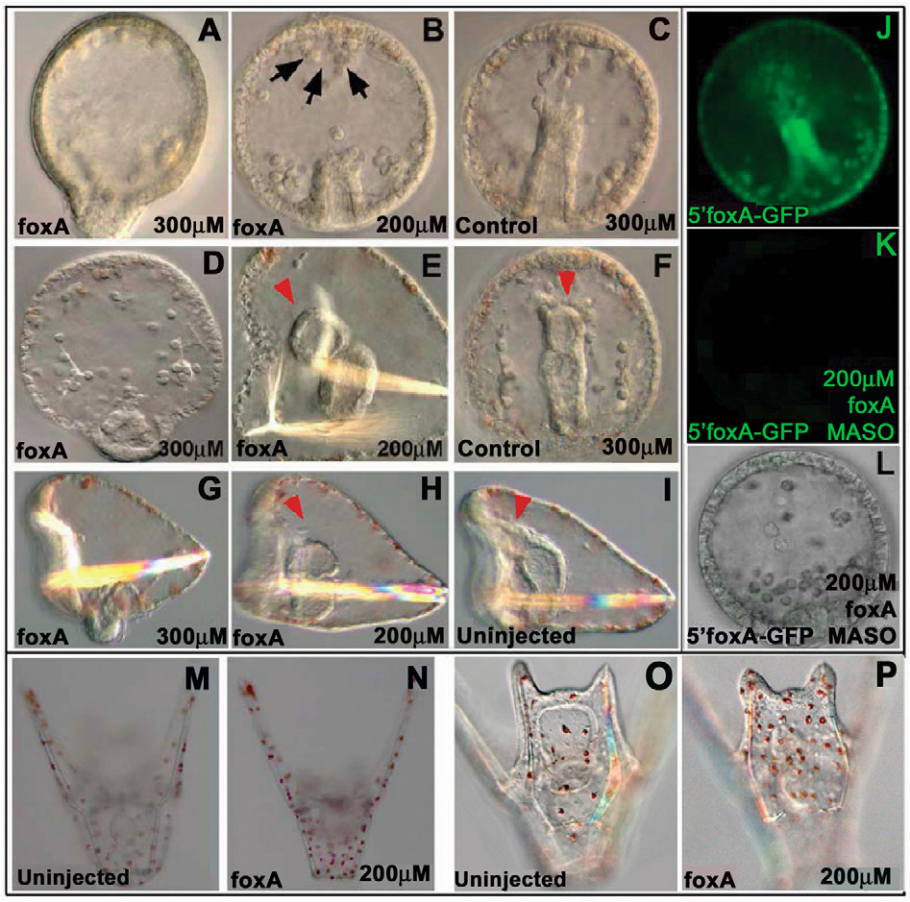

Fig. 3. Effects of *foxa* MASO on development. (**A-L**) *Strongylocentrotus purpuratus* embryos; (**M-P**) *Lytechinus variegatus* embryos. (A-C,J-L) Early-mid gastrula, (D-F) late gastrula, (G-I,M-P) pluteus stage larvae, respectively corresponding to 35 hours, 48 hours and 70 hours of development for *S. purpuratus*. (C,F,I,J,M,O) Control embryos treated as specified in each panel. (A,B,D,E,G,H,K,L,N,P) *foxa* MASO-treated embryos. The concentration of MASO injected is indicated in each panel. Black arrows (B) indicate that the SMCs reach their correct position even if a severe reduction of gut extension has occurred. (J) Fluorescent image of embryos injected with the 5′*foxa*-GFP fusion mRNA: all of the cells express GFP as shown by the (false) green color. (K,L) When *foxa* MASO is co-injected with 5′*foxa*-GFP mRNA embryos do not show any GFP fluorescence (K) but do display the *foxa* MASO phenotype (L). Fluorescent and bright field images of the same embryo. Red arrowheads (E,F,H,I) point to the foregut or to the location where the foregut should be. The increase in pigment cells occurring on MASO treatment can be seen by comparing control embryos (M,O) to MASO-treated embryos (N,P), where the pigment cells appear as dark red cells positioned throughout the aboral ectoderm on the anal side (M,N) and over the oral hood (O,P). The control embryo in O has a mouth, whereas the embryo in P injected with *foxa* MASO does not.

Table 1. Quantitative effects of Foxa knockdown on endomesodermal genes

	Expression*	Blastula†	Mesenchyme blastula†	Early gastrula†	Late gastrula†
foxa	EM, E	+3.1; +1.4‡; NA; NA	+2.0; +1.6; +2.5; +2.1	+1.3‡; +1.4‡; +1.8; NA	+2.5; +1.9; NA; NA
gatae	EM, E	+2.9; +1.8; NA; NA	NS; NS; NS; NS	NS; NS; NS; –1.6	–1.6; –1.6; –2.5; –1.7
endo16	EM, E	NS; +1.6; NA; NA	NS; NS; NS; NS	NS; NS; NS; NS	–2.7; –3.2; –3.3; –2.7
gcm	SMC	NS; NS; NA	NS; NS; NS	+2.2; +1.6; +3.0	NS; NS; NS
hh	E	NE	–5.8; –4.9; –3.4	NA; –2.5; –2.3	–2.7; –3.3; NA

Numbers shown are ΔC_T or normalized C_T differences between *foxa* MASO-injected embryos and control embryos. ΔC_T is calculated as previously described (Oliveri and Davidson, 2004a). A positive number means the number of transcripts of the target gene is increased by the *foxa* MASO; a negative number means the number of target gene transcripts is decreased. Listed data are considered significant when ΔC_T is <–1.6 or >+1.6. Smaller effects are shown as not significant (NS). NE, the gene is not normally expressed at this time point of development and the QPCR result is irrelevant; NA, result not available. Measurements carried out in independent batches of cDNA are separated by semicolon. Each result has been repeated at least in two separate injection experiments. This table shows quantitative results for genes affected by *foxa* knock-down during the developmental time period analyzed. Many other genes are not affected by *foxa* MASO at any timepoints: *otx a, otx β1/2, krl, gatac, hmx, not, nrl, pks, tbr, brn1/2/4, delta, eve, foxc, hox11/13b, krox, wnt8, lox, bra, wnt5, delta, apoL, hnf6, lim1, dri, soxB, foxb, alx1, ets1.*

*Domain of expression in untreated embryos. EM, endomesodoerm; E, endoderm; SMC, secondary mesenchyme cells. For a detailed description of the expression pattern of the genes in the table, see text.

†*S. purpuratus* embryos were collected at 18 hours (blastula), 23-24 hours (mesenchyme blastula), 30-34 hours (early gastrula) and 48 hours (late gastrula) postfertilization.

‡This number, even if below significance, shows the same trend of response to perturbation.

foregut of the embryonic archenteron was truncated or missing altogether (Fig. 3E,H). This region of the archenteron is most sensitive to the absence of Foxa, consistent with the hypothesis that the high level of *foxa* expression in the foregut region during gastrulation is crucial for specification (Fig. 1I,J). At higher concentrations of MASO the whole gut is absent, and in its place is a small, everted, scar-like structure (Fig. 3D,G). In both species, the overall effect of *foxa* MASO treatment is a reduction in the mass of endoderm. Even if invagination occurs, the gut fails to connect with the oral ectoderm and a mouth never forms (Fig. 3P). In addition, MASO-treated embryos produce excess numbers of pigment cells (Fig. 3M,N; an average increase of 40-70%, three experiments, n=10 experimental and 10 control embryos counted at the same stage in each experiment). These data suggest that an absence or reduction of Foxa leads to a diversion of presumptive endodermal cells to mesodermal fate, and a reduction in endoderm specification. The MASO phenotypes are consistent with the expression data shown in Fig. 1, and together with that data confirm *foxa* as a key regulatory gene of the endomesoderm GRN.

The *foxa* gene and the endomesoderm GRN

Table 1 shows the effects, as reflected by QPCR, on genes that execute functions essential to endomesoderm specification. Absence of Foxa function has very specific effects, and the expression of the large majority of genes tested was not quantitatively perturbed in the timeframe studied. The level of *foxa* transcript itself increases sharply when *foxa* mRNA translation is inhibited. Thus, the *foxa* gene is subject to repression by Foxa protein. This result at least partially explains the oscillatory character of the temporal expression profile observed for the *foxa* gene (Fig. 2). An early repressive effect was also seen on *gatae* at the blastula stage, when *foxa* is still transiently expressed in the endomesodermal territory and *gatae* is expressed in territory that will become late SMC mesoderm (Fig. 1) (Lee and Davidson, 2004). By the time the expression of *gatae* occurs in definitive endoderm at mesenchyme blastula stage, there is no evident *foxa* repression of *gatae*. Expression of both *gatae* and *endo16*, a well-known endodermal marker gene, are controlled at this stage by other known endodermal regulators (Yuh et al., 1994) (Table 1). At late gastrula stage in *foxa* MASO-treated embryos, the *gatae* and *endo16* genes show a strong decrease in the level of expression, an obvious consequence of the prior failure of endomesoderm specification (Fig. 3). These last results are not interpreted as evidence for direct regulatory gene interactions in late gastrula, but as a consequence of the earlier failure of Foxa to contribute to endoderm specification. Foxa is also an important regulator of *hedgehog* (*hh*) expression, which is known to be expressed in endoderm beginning just before mesenchyme blastula stage (Walton et al., 2006).

A second important result (see Table 1) is that the early SMC-specific *gcm* gene (Ransick et al., 2002) is revealed to be a target of *foxa* repression in a time window that begins after 24 hours and extends at least until 34 hours. This result provides a direct explanation for the essential role of *foxa* in confining endodermal cells to their appropriate fate, as we demonstrate in the following experiments.

foxa repression is required to exclude mesoderm fate in endoderm cells

foxa MASO reduces gut size or eliminates it, and additional pigment cells are observed (Fig. 3N,P). Because in normal embryos *foxa* is expressed exclusively in veg2 endoderm after very early stages of development, the implication is that a specific function of this gene is to prevent a number of veg2 progeny from executing mesodermal fates. To test this hypothesis directly, a mosaic analysis was performed (see Fig. 4).

The *foxa* MASO was injected into a group of eggs along with Rhodamine dextran. At the 60-cell stage, two fluorescent, MASO-bearing cells from each embryonic tier were transplanted to equivalent positions in place of two normal cells in an unlabeled host embryo, as shown in the diagrams to the left in Fig. 4. These mosaic embryos were then compared with the respective mosaic control embryos, in which the fluorescent transplanted blastomeres contained no MASO. As shown in Fig. 4, control and *foxa* MASO animal tier, veg1 and micromeres cells behaved identically in the host embryos. However, the fate of *foxa* MASO-injected veg2 cells was very different from that of their controls. Transplanted veg2 blastomeres unable to generate the Foxa protein produced only dispersed mesodermal cell types, whereas the control dye-injected veg2 cells became normal gut endoderm, as well as mesodermal cells. This same outcome was observed both in embryos of *S. purpuratus* and *L. variegatus*; 24 cases of each tier in each species were scored. Some experimental veg2 transplant embryos had a few fluorescent cells in the gut, but these were always far fewer in number than in control archenterons, and these often differentiated as ectopic pigment cells or ectopic coelomic pouches, both SMC derivatives. Thus, one function of the *foxa* gene is to repress mesodermal specification in the subset of veg2 cells normally fated to generate gut endoderm.

Further evidence of mesoderm fate exclusion: *foxa* MASO effects on spatial gene expression patterns

WMISH was carried out on *foxa* or control MASO-injected embryos at blastula (data not shown), mesenchyme blastula, early gastrula and late gastrula stages (18 hours, 23-24 hours, 30-34 hours and 48 hours postfertilization for *S. purpuratus*), along with non-injected controls. The genes targeted in this series of experiments were those implicated as being downstream targets of *foxa* in the QPCR experiments (see Table 1), namely *foxa* itself and the mesoderm regulator *gcm* (Ransick et al., 2002). In addition, we looked at *gatae* expression as an indicator of endoderm regulatory state. Representative WMISH results are reproduced in Fig. 5. As expected (Table 1), *gatae* expression appeared normal until the onset of gastrulation (data not shown). As the MASO phenotype indicates, the *foxa* gene is required as a positive regulator of later endoderm development (Fig. 5C,F). Even though gastrulation of *foxa* MASO-treated embryos was delayed or did not occur at all, they produced a higher level of *foxa* transcripts than did controls (Fig. 5G-L), just as had been demonstrated by the QPCR data (Table 1). As indicated earlier, one MASO effect is the maintenance of a high level of *foxa* transcripts in the veg2 cells, by cancellation of the *foxa* autorepression circuit.

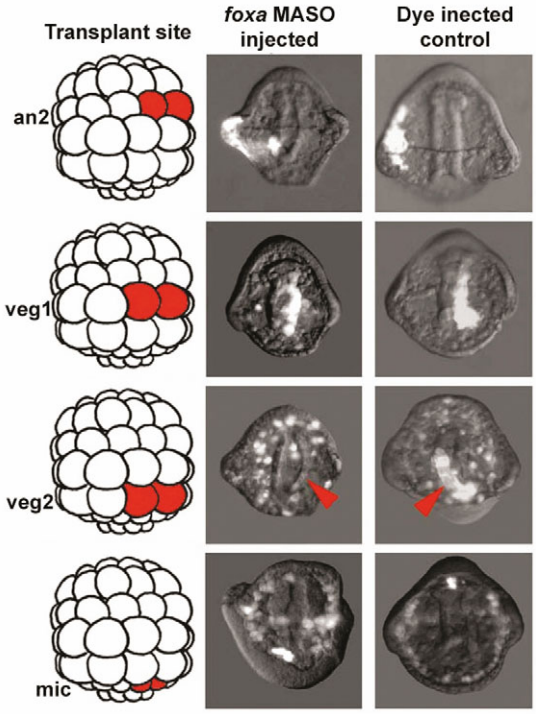

Fig. 4. Mosaic analysis of the fates of specific blastomeres bearing *foxa* MASO. *L. variegatus* eggs were injected with *foxa* MASO and co-injected with rhodamine dextran as a dye tracer. Control donor embryos were injected with rhodamine dextran alone. At the 60-cell stage, two injected cells from each tier were transplanted to host embryos as indicated on the left diagrams. (**Top row**) Cells of the 'an2' tier normally form ectoderm and *foxa* MASO-injected cells behaved like controls. (**Second row**) veg1 cells normally contribute to hindgut, midgut and vegetal ectoderm, and the *foxa* MASO-injected veg1 cells also contributed to gut and ectoderm. (**Third row**) veg2 cells normally contribute to foregut, midgut and SMCs. The *foxa* MASO cells contributed almost exclusively to SMCs. The red arrowhead points to the gut in both control and experimental embryos. (**Bottom row**) *foxa* MASO and control micromeres became normal PMCs.

The reallocation in *foxa* MASO embryos of endodermal cells to a mesodermal state is also visualized by WMISH, by expression of *gcm*. No difference from controls is observed until 24 hours (Fig. 5A,D). By the beginning of gastrulation (32 hours), there is an expansion in the number of cells expressing *gcm* in MASO-treated embryos (Fig. 5B,E; 33% more *gcm* positive cells, n=6 experimental embryos and 6 controls). This is consistent with the derepressive

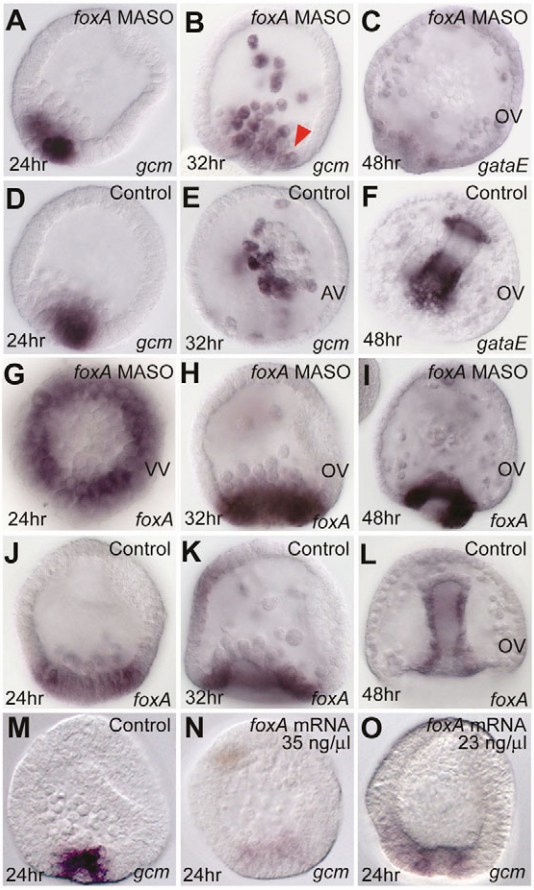

Fig. 5. Spatial expression of endomesoderm genes in *foxa* perturbed embryos. (**A-O**) *S. purpuratus* embryos at different time points injected with *foxa* (A-C,G-I) and control MASO (D-F,J-L) or mRNA (M-O) were hybridized with WMISH probes as indicated in the lower right corner of each panel; developmental time is indicated at lower left. Embryos are in lateral view with the vegetal pole towards the bottom of each panel, unless specified otherwise. AV, view from the apical plate; VV, view from the vegetal plate; OV, view from the oral ectoderm. An identical pattern of *gcm* expression is observed at mesenchyme blastula stage, in *foxa* MASO (A) and control (D) embryos. At 32 hours postfertilization, more cells stained positively for *gcm* are observed in the *foxa* MASO-injected embryo (B) than in control (E, see text for quantitation). The red arrowhead indicates a cell of the invaginating endoderm that is expressing *gcm*. (C,F) *gatae* expression at 48 hours in *foxa* MASO and control (late gastrula) embryos. At this stage, *gatae* is normally expressed in midgut, hindgut and coelomic pouches (Lee and Davidson, 2004); expression is nearly abolished in Foxa-depleted embryos (C). (G-L) Expression of *foxa* in *foxa* MASO and control embryos. At all three stages shown, the intensity of expression is increased by *foxa* MASO (G-I), compared with controls (J-L). The normal expression boundaries are maintained, however; although the remaining endoderm at 48 hours has failed to invaginate. (N,O) mRNA overexpression of Foxa. Complete repression of *gcm* relative to control embryos (M) is seen only at high concentrations (N) of injected mRNA.

effect of *foxa* MASO on *gcm* expression seen by QPCR (Table 1). Furthermore, ectopic *foxa* mRNA overexpression (Fig. 2), depresses the expression of *gcm* (Fig. 5M-O) in a concentration-dependent manner. Only high concentrations of injected mRNA completely abolish *gcm* expression to a level equivalent to the endogenous spike of *foxa* expression.

foxa is required for repression of mesoderm specification in veg2 endoderm only

In normal embryos, the mesoderm territory arises from those veg2 cells that are exposed to a Delta signal expressed in the PMCs during cleavage (McClay, 2000; Sherwood and McClay, 1999; Sweet et al., 2002). The Delta signal is transduced by the Notch (N) receptor in these cells. For early SMC specification, a primary regulatory gene target of N signaling is *gcm*. The genomic interaction is direct, as Su(H) target sites that mediate these N effects have been demonstrated in the cis-regulatory apparatus of the *gcm* gene (Ransick and Davidson, 2006). At the beginning of the mesenchyme blastula stage, the *delta* gene is expressed in the SMCs, and this later signal is received by both late-specified SMCs (Sweet et al., 2002) and veg2 endoderm (Peterson and McClay, 2005). It is reasonable to suppose, therefore, that *foxa* repression of mesoderm fates in the endoderm is required to preclude the same response to N signaling in the endoderm. Embryo recombinants were designed as tests of this proposition.

We investigated whether the *foxa* MASO could produce a transformation to mesodermal fates in veg1 endoderm, as these cells are not exposed to Delta-Notch signaling. At the 60-cell stage, fragments from embryos injected with Rhodamine dextran and *foxa* MASO, were recombined with control embryo fragments, labeled with fluorescein (Fig. 6A,D). Veg1 plus the animal hemisphere of *foxa* MASO embryos were combined with control veg2 plus micromere fragments, and the reciprocal recombinations were also generated. When the veg2 tier contained the *foxa* MASO, excess SMCs were produced by this tier, and there was very little veg2 contribution to the gut (Fig. 6A-C). Instead, the control veg1 cells made up almost all of the gut, even the foregut, which normally receives almost no contribution from the veg1 descendants. The foregut patches in Fig. 6B,C (red) were observed frequently, and much of that tissue became mesodermal coelomic pouches. Essentially, this is the same as the result shown in the mosaics (Fig. 4). In the reciprocal combination (Fig. 6D-F), in which all veg1 cells contained the *foxa* MASO, most of the gut is derived from the control veg2 cells (green). Thus, there is a subnormal contribution of veg1 cells (red) to the gut. The *foxa* MASO-injected veg1 cells contributed a small amount of gut in some cases (the MASO-treated cells produced a subnormal amount of hindgut, see Fig. 6E,F), but in each case the control veg2 cells regulated to compensate for the reduced endoderm formed by *foxa*-MASO veg1 cells. It follows that, in the veg1 cells, the *foxa* gene contributes to postgastrular specification of the endoderm, as, in its absence, far fewer veg1 cells become hindgut and midgut endoderm. Notably, no excess SMC types of veg1 origin were observed. Veg1 cells do not receive the Delta-Notch signal, and therefore one function employed by Foxa in veg2 cells (*gcm* repression) is not detectable in veg1 cells.

Formation of the mouth requires *foxa* gene expression in the oral ectoderm

The *foxa* gene is expressed in the oral ectoderm region, beginning at about 24 hours after fertilization (Fig. 1), and, in the whole embryo, *foxa* MASO precludes formation of the mouth (Fig. 3). To confirm directly that oral expression of *foxa* is necessary for mouth formation, chimeric recombinations were generated (see Fig. 7). Recombinants were produced at the 32- to 60-cell stage. Uninjected halves (green) were reciprocally recombined with *foxa* MASO-injected halves (red; see Fig. 7A,D). The embryos were imaged at 40 hours. Embryos with control (green) animal halves had a normal mouth (Fig. 7C). Embryos without Foxa in the animal half (Fig. 7E,F) make a normal gut but no mouth. Thus, the oral territory of *foxa* expression is indeed required for production of the mouth.

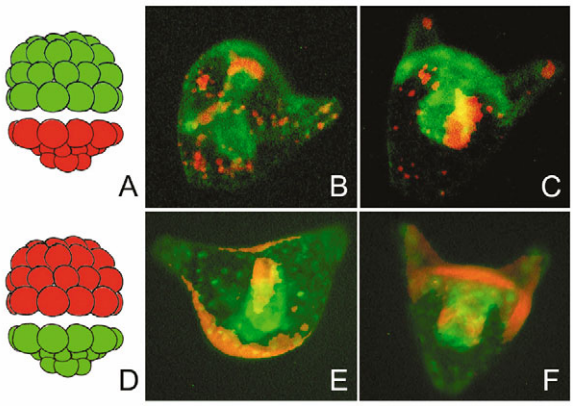

Fig. 6. Veg1 contributions in recombinant embryos bearing *foxa* MASO. (**A**,**D**) Diagram of recombinants consisting of an animal half plus veg1 (top part), recombined with veg2 plus micromeres (lower part). Control *L. variegatus* embryos were dyed green with fluorescein dextran, and experimental embryos were injected with *foxa* MASO and co-injected with RITC (red) before recombination. (**B**,**C**,**E**,**F**) In the recombinants, the control (green) endoderm from veg 1 (B,C) or from veg 2 (E,F) makes much of the gut; this is dependent upon whether veg2 (B,C) or veg1 (E,F) contains *foxa* MASO. Recombinations were made at the 60-cell stage. (B,C) Pluteus stage chimeras as in A. (E,F) Pluteus stage chimeras as in D. See text for interpretation.

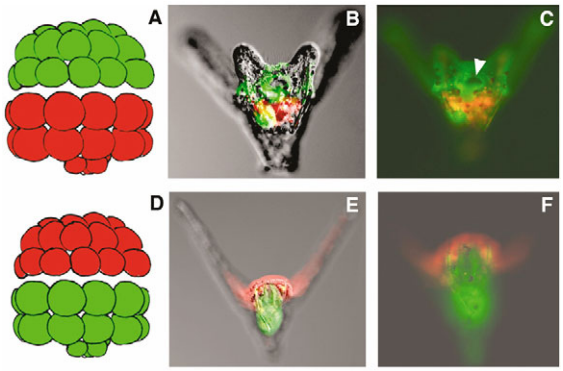

Fig. 7. Oral expression of *foxa* is required for mouth formation. Red indicates *foxa* MASO-injected cells; green indicates control cells. (**A**,**D**) Diagram of the experiment. Reciprocal combinations of animal and vegetal halves were recombined. (**B**,**C**) Control animal half, showing normal mouth (arrowhead, C). The *foxA* MASO vegetal half (red) produces a truncated gut, with most of the red cells becoming SMCs, and extra pigment cells (compare black cells in C and F). (**E**,**F**) *foxa* MASO-injected animal half (red) produces ectoderm with no mouth. The control vegetal half (green) produces a normal gut, including foregut that leads to a dead end. (B,E) DIC images superimposed on fluorescent images. (C,F) Fluorescent image only at a slightly higher magnification, focused on the mouth region.

DISCUSSION

We have shown that the *foxa* regulatory gene executes three different classes of function in the development of the sea urchin embryo. Only the first of these can, at present, be understood in mechanistic detail, as only this function takes place in the context of the established endomesoderm GRN: *foxa* represses *gcm* and mesoderm specification in the presumptive endoderm of the mesenchyme blastula-early gastrula embryo. The two other roles of *foxa*, later specification of endoderm and specification of a mouth in the oral ectoderm, occur later in the postgastrular embryo, which has yet to be analyzed at the gene regulatory network level. Foxa involvement in endoderm specification has been observed in all bilaterarians examined to date. In addition, expression of *foxa* is necessary for development of the stomodeum, a new observation.

The participation of a given regulatory gene in multiple developmental processes, controlled by separate GRNs, is an emergent theme in regulation molecular biology. From an external point of view, this is an obvious and general requirement stemming from the fact that all bilaterians use essentially the same regulatory gene toolkit, so that in their immensely diverse developmental processes the same genes have to be deployed over and over again for different purposes (for reviews, see Davidson, 2006; Erwin and Davidson, 2002). Furthermore, in the sea urchin embryo, 80% of all regulatory genes are used just to get to the late gastrula stage (Howard-Ashby et al., 2006), and multiple re-use of these genes is therefore an inescapable inference. Many particular examples of regulatory genes that execute totally distinct functions during sea urchin embryogenesis are indeed already in hand, for example *hnf6* (Otim et al., 2004), *otx* (Li et al., 1997; Yuh et al., 2002), *deadringer* (Amore et al., 2003), *gsc* (Angerer et al., 2001), and *blimp1/krox* (Livi and Davidson, 2006a; Livi and Davidson, 2006b). The *C. elegans* ortholog of the *foxa* gene, *pha4*, regulates different gene batteries at different times (Ao et al., 2004; Gaudet and Mango, 2002; Gaudet et al., 2004), and a particular aspect of its multiple capabilities is that the Foxa transcription factor recognizes high and low affinity target sites according to its concentration. That the same is likely to be true for sea urchin *foxa* is implied by the oscillatory time course shown in Fig. 2. This is clearly due to *foxa* autorepression (Table 1). But when the autorepression is blocked from occurring by the introduction of *foxa* MASO, there is no change in the location of expression (Fig. 5), so the only significance of the normal oscillation is to alter the concentration of the *foxa* gene product over time within the cells of the endoderm. Where the specific targets of *foxa* are known, i.e. in the early phase of its function, we can associate the level of *foxa* expression with a given function, and from the time course of the oscillation phase we can determine the temporal duration of that function.

The *foxa* gene in the endomesoderm GRN

During the blastula stage, *foxa* has no input into any known portion of the regulatory apparatus controlling endoderm specification (Table 1; genes not affected by *foxa* MASO), nor does the blockade of Foxa translation cause any digression from normal developmental morphology up to gastrulation. But nonetheless, this gene has a function that is essential for endoderm specification, namely, to permit this specification to occur at all. Fig. 8A shows the inputs that activate *foxa* in the veg2 endoderm. Following mesoderm specification, the *delta* gene becomes active in mesoderm cells (Fig. 8C), although this second phase of Delta expression is independent of the first PMC Delta signal (Sweet et al., 2002). The second signal is received in the adjacent veg2 cells, where it is essential for activation of the essential endoderm regulatory gene *gatae*. This has been shown to be a direct cis-regulatory input mediated by the Su(H) transcription factor (P. Y. Lee and E.H.D., unpublished). As the *gcm* gene is directly activated by N signaling via Su(H) as well (*op cit*), and because *foxa* expression in the endoderm normally represses *gcm* (Table 1, Fig. 5), the effect of preventing *foxa* expression is to promote ectopic *gcm* expression in cells that normally would become endoderm. These cells now become respecified as mesodermal cells (Figs 4, 6). In other words, a means of preventing *gcm* expression in endoderm is essential as a device to permit endoderm specification resulting from the N input.

Seen in this light, the fine-tuned elegance of the *foxa* regulatory architecture (Fig. 8) is thought provoking when viewed in evolutionary terms. Both PMCs and precociously specified pigment cells are echinoid specialties, and so at least parts of this architecture are likely to have been installed since the divergence of euechinoids. In starfish, there is also a N input into *gatae* in the endoderm at the equivalent stage, and *foxa* also represses itself, but *gcm* is expressed quite differently and in cells arising elsewhere (Hinman et al., 2003) (V. F. Hinman and E.H.D., unpublished). The *foxa* repression function has different targets in the starfish, including *gatae* in the mesoderm (Hinman et al., 2003). Since divergence, not only has *foxa* repression of *gcm* in the endoderm been inserted in the euechinoid lineage, but its operation is temporally regulated by its own autorepression so as to operate at just the right time to control N signal effects; high levels of *foxa* gene product are apparently required for both *gcm* repression and *foxa* autorepression.

Notch signaling is directly or indirectly required for specification of the late delaminating SMC derivatives, muscle and coelomic pouches (Sweet et al., 2002; Oliveri et al., 2002; Peterson and McClay, 2005; Sherwood and McClay, 1999). The precursors of these cells are sorted out within the SMC domain during the blastula stage (Ruffins and Ettensohn, 1996), when *foxa* is transiently expressed in an overlapping domain with *gcm*. Because N signaling activates the *gcm* gene, and indeed is the necessary input required earlier to activate *gcm* in the mesoderm cells in response to the initial Delta signal received from the PMCs (Ransick and Davidson, 2006), and because Foxa represses *gcm*, the early phase of *foxa* expression could affect fate allocation among SMCs as well. In this case, some of the excess pigment cells produced by *foxa* MASO treatment would reflect an alteration of SMC fate balance.

Later functions of *foxa*

Only phenotypical evidence suggests later roles of *foxa*, and no direct gene targets in either the archenteron or the stomodeal area are demonstrated. Furthermore, it must be considered whether the effects of *foxa* MASO treatment on development of the archenteron (see Fig. 3A-I, Fig. 5A-F) could, at least in part, just be secondary effects of the conversion of veg2 cells to SMC fates. Alternatively, convincing arguments suggest that *foxa* directly promotes the gene expression required for further development of the archenteron. The postgastrular roles of this gene are most simply interpreted as the provision of positive inputs into gut and stomodeal genes, even though the pregastrular role, the only one of which we know the mechanism, is a repressive one. It is possible that the amplitude of expression seen in Fig. 2 is permanently damped after gastrulation gets underway, and, as we speculate above, perhaps Foxa acts as a repressor only when expressed at high levels. This would explain the autorepression revealed by increased *foxa* production in the presence of *foxa* MASO, and the repression effect on *gcm* expression only at a time when endogenous *foxa* is at its highest levels.

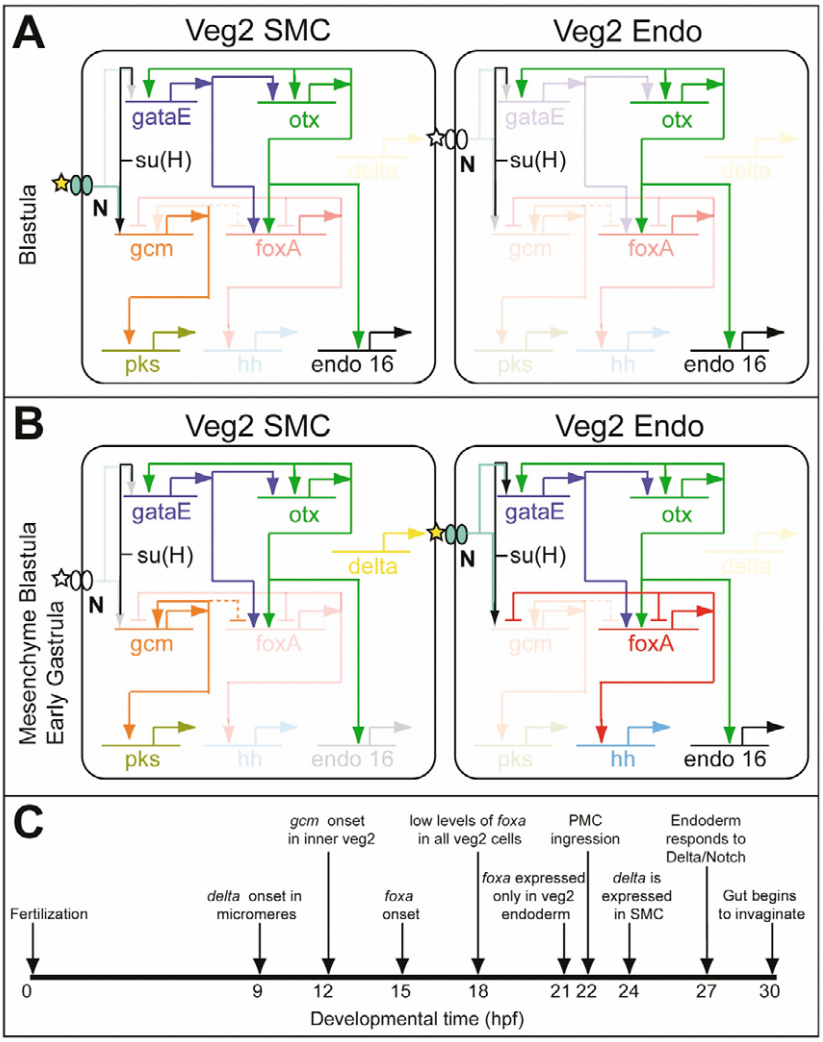

Fig. 8. *foxa* gene interactions in the endomesoderm regulatory network. Relevant network subcircuits are depicted as 'views from the nuclei' (Bolouri and Davidson, 2002) up to beginning of gastrulation in veg2 SMC (left boxes) and veg2 endodermal cells (right boxes). Architecture is as presented previously (Davidson, 2006; Erwin and Davidson, 2002; Levine and Davidson, 2005; Oliveri and Davidson, 2004b) (for a current version see http://sugp.caltech.edu/endomes/), with the addition of data from this paper. (**A**) Blastula; (**B**) mesenchyme blastula to early gastrula. Solid connecting lines are predicted to be direct interactions between the product of a given regulatory gene and the target cis-regulatory module of other gene(s). The dashed line is an implied and possibly indirect interaction. Arrows indicate activating interactions and bars indicate repressive inputs. Bold colors are genes expressed or interactions operating in that cell type at that time; faded colors indicate genes that are inactive or interactions not then taking place. Yellow stars symbolize the Delta ligand and turquoise ovals the Notch receptor embedded in the diagrammatic membrane. White stars and white ovals indicate that Delta/Notch signaling is not active at a particular stage in a particular cell type (because the *delta* gene is not expressed there). At blastula stage (A), the genes *gataE* (blue) and *otx* (green) enter into a feedback-stabilizing loop (Yuh et al., 2004), and are responsible for initiating expression of the *foxa* gene (red) in all endomesodermal cells. In SMCs only, the *gcm* gene (orange) is activated in response to the Delta signal arriving from the adjacent micromeres (Sherwood and McClay, 1997; Sherwood and McClay, 1999). Gcm is then responsible for activating the pigment cell program represented by the *pks* gene (olive green). By mesenchyme blastula stage (B), *gataE* is expressed in veg2 endoderm as well as in mesoderm (Lee and Davidson, 2004). *foxa* expression becomes very strong and is restricted to endodermal cells. High levels of *foxa* are responsible for autorepression of *foxa*, and for repression of *gcm*. In the *gcm* expressing SMCs, *foxa* expression fades out; an unknown function downstream of *gcm* is inferred. The mesodermal cells now begin to express the Delta ligand that will signal to the adjacent veg2 endodermal cells, where Notch signal is used to drive the expression of *gataE* (P. Y. Lee and E.H.D., unpublished). At this time, *gcm* expression in response to Delta/Notch signaling is prevented in veg2 endoderm by the repressive action of Foxa. (**C**) Timeline of expression of the components in the model based on QPCR and WMISH data.

The conversion to an SMC fate affects only veg2 and not veg1 endoderm. In the absence of veg2 endoderm, or if veg2 is unable to express *foxa*, veg1 endoderm can form the entire gut. The chimera experiments (Fig. 6) show clearly that when veg1 contains the *foxa* MASO it fails to generate hindgut endoderm or contribute at all to the midgut, as it does in normal embryos (Logan and McClay, 1997; Ransick and Davidson, 1998). But because veg1 is not subject to conversion to SMCs (because it has not received the Notch signal), the *foxa* MASO must interfere with other functions of *foxa* needed in the postgastrular endoderm of the hindgut. Note that *foxa* is normally expressed in the late gastrula in all of the gut (Fig. 1). It is likely that the MASO effects on foregut development are also due to the failure of regulatory interactions needed for that aspect of gut morphogenesis and differentiation, this time in veg2 derivatives. These deductions predict postgastrular GRN subcircuits in which the *foxa* gene, no doubt together with other regulators, operates batteries of downstream genes that are required in the anterior and posterior domains of the gut, respectively.

We thank Dr Andy Ransick of California Institute of Technology for critical and fruitful discussions; Jina Yun whose extraordinary technical assistance made the network analysis as complete as possible; Drs Qiang Tu and Özlem Yüce for help with the WMISH. We acknowledge Dr Cathy Yuh, who generously provided the *S. purpuratus* clone; Dr Albert Erives, who designed the *foxa* MASOs and screened the *S. purpuratus* BAC library; Dr Jenifer Croce, who provided probes and offered many suggestions on Notch signaling; and Dr Jim A. Coffman for the cDNA sequence of *foxa*. This research was supported by NIH grants HD37105, HD14483, GM61464, and by the Office of Science (BER), the US Department of Energy, grant DE-FG02-03ER63584. P.O. was supported by the Camilla Chandler Frost Fellowship; K.D.W. by the US Department of Defense.

References

Amore, G., Yavrouian, R. G., Peterson, K. J., Ransick, A., McClay, D. R. and Davidson, E. H. (2003). Spdeadringer, a sea urchin embryo gene required separately in skeletogenic and oral ectoderm gene regulatory networks. *Dev. Biol.* **261**, 55-81.

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development* **119**, 1301-1315.

Angerer, L. M., Oleksyn, D. W., Levine, A. M., Li, X., Klein, W. H. and

Angerer, R. C. (2001). Sea urchin goosecoid function links fate specification along the animal-vegetal and oral-aboral embryonic axes. *Development* **128**, 4393-4404.
Ao, W., Gaudet, J., Kent, W. J., Muttumu, S. and Mango, S. E. (2004). Environmentally induced foregut remodeling by PHA-4/FoxA and DAF-12/NHR. *Science* **305**, 1743-1746.
Bolouri, H. and Davidson, E. H. (2002). Modeling DNA sequence-based cis-regulatory gene networks. *Dev. Biol.* **246**, 2-13.
Corbo, J. C., Erives, A., Di Gregorio, A., Chang, A. and Levine, M. (1997). Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. *Development* **124**, 2335-2344.
Davidson, E. H. (2006). *The Regulatory Genome: Gene Regulatory Networks in Development and Evolution*. San Diego, London: Academic Press.
Davidson, E. H., Rast, J. P., Oliveri, P., Ransride, A., Calestani, C., Yuh, C. H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C. et al. (2002). A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. *Dev. Biol.* **246**, 162-190.
Erwin, D. H. and Davidson, E. H. (2002). The last common bilaterian ancestor. *Development* **129**, 3021-3032.
Fritzenwanker, J. H., Saina, M. and Technau, U. (2004). Analysis of forkhead and snail expression reveals epithelial-mesenchymal transitions during embryonic and larval development of Nematostella vectensis. *Dev. Biol.* **275**, 389-402.
Gaudet, J. and Mango, S. E. (2002). Regulation of organogenesis by the Caenorhabditis elegans FoxA protein PHA-4. *Science* **295**, 821-825.
Gaudet, J., Muttumu, S., Horner, M. and Mango, S. E. (2004). Whole-genome analysis of temporal gene expression during foregut development. *PLoS Biol.* **2**, e352.
Harada, Y., Akasaka, K., Shimada, H., Peterson, K. J., Davidson, E. H. and Satoh, N. (1996). Spatial expression of a forkhead homologue in the sea urchin embryo. *Mech. Dev.* **60**, 163-173.
Hinman, V. F., Nguyen, A. T., Cameron, R. A. and Davidson, E. H. (2003). Developmental gene regulatory network architecture across 500 million years of echinoderm evolution. *Proc. Natl. Acad. Sci. USA* **100**, 13356-13361.
Howard-Ashby, M. L., Materna, S., Oliveri, P., Tu, Q., Brown, C. T. and Davidson, E. H. (2006). The "regulome" requirements of embryogenesis in the sea urchin: implications for bilaterian evolution. *Science* (in press).
Kaestner, K. H., Knochel, W. and Martinez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev.* **14**, 142-146.
Lee, P. Y. and Davidson, E. H. (2004). Expression of Spgatae, the Strongylocentrotus purpuratus ortholog of vertebrate GATA4/5/6 factors. *Gene Expr. Patterns* **5**, 161-165.
Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.
Levine, M. and Davidson, E. H. (2005). Gene regulatory networks for development. *Proc. Natl. Acad. Sci. USA* **102**, 4936-4942.
Li, X., Chuang, C. K., Mao, C. A., Angerer, L. M. and Klein, W. H. (1997). Two Otx proteins generated from multiple transcripts of a single gene in Strongylocentrotus purpuratus. *Dev. Biol.* **187**, 253-266.
Livi, C. B. and Davidson, E. H. (2006a). Expression and function of blimp1/krox, an alternatively transcribed regulatory gene of the sea urchin endomesoderm network. *Dev. Biol*. **293**, 513-525.
Livi, C. B. and Davidson, E. H. (2006b). Regulation of spblimp1/krox1a, an alternatively transcribed isoform expressed in midgut and hindgut of the sea urchin gastrula. *Gene Expr. Patterns* (in press).
Logan, C. Y. and McClay, D. R. (1997). The allocation of early blastomeres to the ectoderm and endoderm is variable in the sea urchin embryo. *Development* **124**, 2213-2223.
Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.
Mango, S. E., Thorpe, C. J., Martin, P. R., Chamberlain, S. H. and Bowerman, B. (1994). Two maternal genes, apx-1 and pie-1, are required to distinguish the fates of equivalent blastomeres in the early Caenorhabditis elegans embryo. *Development* **120**, 2305-2315.
Martindale, M. Q., Pang, K. and Finnerty, J. R. (2004). Investigating the origins of triploblasty: 'mesodermal' gene expression in a diploblastic animal, the sea anemone Nematostella vectensis (phylum, Cnidaria; class, Anthozoa). *Development* **131**, 2463-2474.
McClay, D. R. (2000). Specification of endoderm and mesoderm in the sea urchin. *Zygote* **8**, S41.
Minokawa, T., Rast, J. P., Arenas-Mena, C., Franco, C. B. and Davidson, E. H. (2004). Expression patterns of four different regulatory genes that function during sea urchin development. *Gene Expr. Patterns* **4**, 449-456.
Nemer, M., Rondinelli, E., Infante, D. and Infante, A. A. (1991). Polyubiquitin RNA characteristics and conditional induction in sea urchin embryos. *Dev. Biol.* **145**, 255-265.
Oliveri, P. and Davidson, E. H. (2004a). Gene regulatory network analysis in sea urchin embryos. *Methods Cell Biol.* **74**, 775-794.
Oliveri, P. and Davidson, E. H. (2004b). Gene regulatory network controlling embryonic specification in the sea urchin. *Curr. Opin. Genet. Dev.* **14**, 351-360.
Oliveri, P., Carrick, D. M. and Davidson, E. H. (2002). A regulatory gene network that directs micromere specification in the sea urchin embryo. *Dev. Biol.* **246**, 209-228.
Oliveri, P., Davidson, E. H. and McClay, D. R. (2003). Activation of pmar1 controls specification of micromeres in the sea urchin embryo. *Dev. Biol.* **258**, 32-43.
Olsen, C. L. and Jeffery, W. R. (1997). A forkhead gene related to HNF-3beta is required for gastrulation and axis formation in the ascidian embryo. *Development* **124**, 3609-3619.
Otim, O., Amore, G., Minokawa, T., McClay, D. R. and Davidson, E. H. (2004). SpHnf6, a transcription factor that executes multiple functions in sea urchin embryogenesis. *Dev. Biol.* **273**, 226-243.
Peterson, R. E. and McClay, D. R. (2005). A Fringe-modified Notch signal affects specification of mesoderm and endoderm in the sea urchin embryo. *Dev. Biol.* **282**, 126-137.
Ransick, A. and Davidson, E. H. (1995). Micromeres are required for normal vegetal plate specification in sea urchin embryos. *Development* **121**, 3215-3222.
Ransick, A. and Davidson, E. H. (1998). Late specification of Veg1 lineages to endodermal fate in the sea urchin embryo. *Dev. Biol.* **195**, 38-48.
Ransick, A. and Davidson, E. H. (2006). cis-regulatory processing of Notch signaling input to the sea urchin glial cells missing gene during mesoderm specification. *Dev. Biol.* **297**, 587-602.
Ransick, A., Rast, J. P., Minokawa, T., Calestani, C. and Davidson, E. H. (2002). New early zygotic regulators expressed in endomesoderm of sea urchin embryos discovered by differential array hybridization. *Dev. Biol.* **246**, 132-147.
Rast, J. P., Amore, G., Calestani, C., Livi, C. B., Ransick, A. and Davidson, E. H. (2000). Recovery of developmentally defined gene sets from high-density cDNA macroarrays. *Dev. Biol.* **228**, 270-286.
Ruffins, S. W. and Ettensohn, C. A. (1996). A fate map of the vegetal plate of the sea urchin (Lytechinus variegatus) mesenchyme blastula. *Development* **122**, 253-263.
Sherwood, D. R. and McClay, D. R. (1997). Identification and localization of a sea urchin Notch homologue: insights into vegetal plate regionalization and Notch receptor regulation. *Development* **124**, 3363-3374.
Sherwood, D. R. and McClay, D. R. (1999). LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. *Development* **126**, 1703-1713.
Suri, C., Haremaki, T. and Weinstein, D. C. (2004). Inhibition of mesodermal fate by Xenopus HNF3beta/FoxA2. *Dev. Biol.* **265**, 90-104.
Sweet, H., Amemiya, S., Ransick, A., Minokawa, T., McClay, D. R., Wikramanayake, A., Kuraishi, R., Kiyomoto, M., Nishida, H. and Henry, J. (2004). Blastomere isolation and transplantation. *Methods Cell Biol.* **74**, 243-271.
Sweet, H. C., Gehring, M. and Ettensohn, C. A. (2002). LvDelta is a mesoderm-inducing signal in the sea urchin embryo and can endow blastomeres with organizer-like properties. *Development* **129**, 1945-1955.
Taguchi, S., Tagawa, K., Humphreys, T., Nishino, A., Satoh, N. and Harada, Y. (2000). Characterization of a hemichordate fork head/HNF-3 gene expression. *Dev. Genes Evol.* **210**, 11-17.
Tu, Q., Brown, C. T., Davidson, E. H. and Oliveri, P. (2006). Sea urchin forkhead gene family phylogeny and embryonic expression. *Dev. Biol.* (in press).
Walton, K., Croce, J. C., Glenn, T. D., Wu, S.-Y. and McClay, D. R. (2006). Genomics and expression profiles of the Hedgehog and Notch signaling pathways in sea urchin development. *Dev. Biol.* (in press).
Weigel, D., Jurgens, G., Kuttner, F., Seifert, E. and Jackle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell* **57**, 645-658.
Yuh, C. H., Ransick, A., Martinez, P., Britten, R. J. and Davidson, E. H. (1994). Complexity and organization of DNA-protein interactions in the 5′-regulatory region of an endoderm-specific marker gene in the sea urchin embryo. *Mech. Dev.* **47**, 165-186.
Yuh, C. H., Brown, C. T., Livi, C. B., Rowen, L., Clarke, P. J. and Davidson, E. H. (2002). Patchy interspecific sequence similarities efficiently identify positive cis-regulatory elements in the sea urchin. *Dev. Biol.* **246**, 148-161.
Yuh, C. H., Dorman, E. R., Howard, M. L. and Davidson, E. H. (2004). An otx cis-regulatory module: a key node in the sea urchin endomesoderm gene regulatory network. *Dev. Biol.* **269**, 536-551.